

Study Protocol And Statistical Analysis Plan

DINAMIC

Version: 1

Author: Paola Vitaglione, University of Naples, Federico II

Date: 01/30/2017

Title: Diet-induced Arrangement of the Gut Microbiome for Improvement of Cardiometabolic Health (DINAMIC)

Principal Investigator: Paola Vitaglione, University of Naples, Federico II

1. Study Objectives

Mediterranean diet (MedD) has been recognized as an intangible cultural heritage by UNESCO and was shown to be beneficial for the treatment of obesity, type-2 diabetes and cardiovascular diseases. Individuals with the highest adherence to Med-D (classified according to Sofi et al., 2010) were characterized by increased levels of specific fibre-degrading bacteria, increased faecal levels of short chain fatty acids, and lower urinary concentrations of the atherogenic compound TMAO. However, the interplays between Med-D and microbial populations in the intestine remain unclear. Moreover, a number of clinical conditions like obesity, T2D, and atherosclerosis are associated with dysbiotic microbial ecosystems in the gut, i.e., shifts in the structure and function of the microbiota, but the characteristic features of dysbiotic gut communities and the impact of diet are not very well defined. The present study will evaluate the impact of MedD on cardiometabolic health in human subjects via modification of intestinal microbial communities and its impact on health outcomes, mainly related to inflammatory, oxidative and hormonal status. Therefore 8-weeks randomized controlled intervention study will be conducted in healthy subjects with low consumption of fruits and vegetables (and therefore not adhering to the Mediterranean diet) and a low level of physical activity.

1. *Primary aim:* to evaluate the effect of the Mediterranean diet on the inflammatory state and serum lipid profile as well as gut fermentation (assessed by fecal short chain fatty acid, SCFA) of healthy subjects with unhealthy eating habits and a sedentary lifestyle.
2. *Secondary aims:* to evaluate the effect of the Mediterranean diet on microbiome composition and activity, plasma endocannabinoids concentration, gastro-intestinal hormones concentration, serum and urinary polyphenols concentration.

2. Research Design and Procedures

1. Sample Size Justification

The sample size was calculated considering as primary endpoints fasting blood cholesterol, urinary ferulic acid and fecal SCFA.

A sample size of 26 participants would be adequate to detect a 10% change in fasting total cholesterol by using variation in accordance with previous studies (Annuzzi et al., 2014; Vitaglione et al., 2015). Moreover, 33 subjects would be sufficient to detect a 50% change in urinary ferulic acid using inter-subjects variability found in a previous study from our research group where 30 subjects were also found sufficient to detect a 3% increase of *Prevotella* (Vitaglione et al., 2015).

The sample size needed to detect an effect of MedD on individual levels of fecal SCFA (acetate, propionate, butyrate) was calculated considering that in a previous study 6 subjects were sufficient to detect a difference of 20% between groups with a low vs high adherence of MedD (De Filippis et al., 2016). Therefore 40 participants for each treatment group would be sufficient to detect a significant effect of MedD on biomarkers of gut fermentation as well as on modification of gut microbiota composition with an α error of 0.05, 80% power, and 2-sided testing.

b. Subject Population

Eighty people, of both sexes, will be recruited to participate in this study. Participants' ages will range from 18-65 years and will report being healthy. The selection of volunteers will be carried out by a nutritionist and a doctor and includes:

- 1) collection of information concerning personal data, work activity and lifestyle through a prepared form. In addition, the collection of anamnestic data, including alcohol (quantity and type of drink) and/or medication consumption, is scheduled;
- 2) assessment of nutritional status by calculating the body mass index (BMI).
- 3) evaluation of the composition of the habitual diet through a food consumption frequency questionnaire (FFQ) and the 7-day food diary;

The eligibility of the volunteers to participate in the study is defined on the basis of the inclusion and exclusion criteria listed below.

Inclusion Criteria

- Healthy subjects;
- $20 \leq \text{age} \leq 65$ years;
- $\text{BMI} \geq 24 \text{ kg / m}^2$;
- Both sexes;
- No consumption of probiotics and functional foods and / or food supplements of any kind;
- Habitual diet characterized by no more than 2 portions a day of whole foods and / or enriched with dietary fiber;
- Habitual diet with no more than 3 servings of fruit and vegetables per day;
- Low level of physical activity (sedentary life);
- Signature of the informed consent form.

Exclusion Criteria

- Gastrointestinal disorders of any kind;
- Pregnancy or breastfeeding;
- Previous abdominal surgery;
- Hypertriglyceridaemia (Triglycerides > 300 mg / dL);
- Hypercholesterolemia (Cholesterol > 220 mg / dL);
- Arterial hypertension;
- Pharmacological treatments of any type at enrollment and in the 2 months prior to the study;
- Habitual diet rich in fruit and vegetables;
- High level of physical activity;
- Consumption of alcohol equivalent and / or greater than 3 glasses of wine per day;
- Contemporary participation in other studies.

c. Identification and Recruitment of Subjects

Volunteers will be recruited at the Department of Agricultural sciences and the Department of Clinical Medicine and Surgery of the University of Naples "Federico II" among staff members, relatives and friends. Subjects will be recruited into the study also by public announcements, local newspaper and through social networks.

d. Procedures

The study will take place at the Department of Agricultural Sciences and the Department of Clinical Medicine and Surgery of the "Federico II" University of Naples with the participation of nutritionists, doctors and microbiologists. The nutritionist will explain to the selected subjects the design of the study, the rationale and submit them the information for the subject, the informed consent form and the consent to the processing of personal data form. Subjects will have all the time necessary to read and decide whether to accept or refuse to participate in the study. Enrolled subjects will have a 2-week run-in period where they will be asked to not change their habitual diets and physical activity. After that they will be randomly assigned to the Mediterranean Diet (MedD) or Control Diet (ConD) intervention group. Randomization will be done by using a randomization list previously settled. The subjects assigned to the MedD will undergo an individual dietary intervention specifically designed to respect the principles of the Mediterranean diet while keeping the daily energy income unchanged. Each volunteer will receive indications on the type, weekly frequency and quantity of food to consume and those to eliminate or reduce. Subjects assigned to the ConD will continue with their usual diet throughout the duration of the study. Each nutritional intervention will last 8 weeks. All subjects will maintain physical activity unchanged throughout the duration of the study.

Every 2 weeks the volunteers will be subjected to a telephone interview to assess food frequency consumption and physical activity level during the previous week in order to verify the progress of the protocol, to renew the indications and to collect any news / requests from the volunteers.

On the days before and after 4 and 8 weeks from the beginning of the intervention, fasting (from 12 hours) volunteers, will reach the blood sampling room of the San Ciro Diagnostic Center (via Libertà, 270, Portici, if enrolled at the Department of Agricultural sciences) or the diabetes ambulatory of the Department of Clinical Medicine and Surgery (if enrolled there). There, subjects will be checked for the health condition at the moment and over the previous week. Those who will declare to feel well, were subjected to collection of biological samples such as blood, saliva and urine samples as well as stool sample from the previous day; the others will have the visit postponed. Moreover, blood glucose on capillary blood (by finger prick and portable glucometer), blood pressure, body weight, waist and hips circumferences as well as body composition by bioimpedance analysis, will be measured. A dietician will check the 7 days-food diaries compiled during the previous week and subjects will fill the physical activity questionnaires.

3. Data Collection

The only identifiable information will be collected through the informed consent document. The informed consent documents (hard copy), which include the participant's assigned identifier, will be stored in a locked cabinet in the PI's office located in the Agricultural Sciences Department at P.co Gussone Ed.84, 80055, Portici (Naples, Italy).

These documents will be separated from the others and only the PI will have access to them. All data will be coded using a personal identifier assigned to the participant after informed consent is signed, thus all collected data will be coded. All the samples will contain only the participant ID number, time of collection, and session date; thus, participants will not be able to be identified. The investigators will guarantee the confidentiality of the information, which will be analyzed anonymously. At their request, the subjects participating in the study will be able to read their own form and at any time they can withdraw their consent and all the documentation concerning their state of health, even without any motivation.

4. Sample/Specimen Collection

The collection of blood samples will be performed using BD Vacutainer™ tubes by a professional nurse in presence of a doctor at the 'Diabetology clinic of the Department of Clinical Medicine and Surgery of the University "Federico II" of Naples or in a infirmary at the Centro Diagnostico San Ciro Srl , via Libertà, 270, Portici (Naples, Italy). Within 30 minutes of collection, samples will be centrifuged at a speed of 1400 rpm for 10 min at 4 ° C and the obtained plasma and serum samples will be aliquoted into 500 µL tubes. No samples will include identifiable information and the results from assays will be shared between study team members using the participant ID numbers.

5. Data analysis

The design was a parallel group design with repeated measurements over time, in which subjects were randomised between two arms, MedD and ConD. Data will be expressed as the mean±SEM, unless otherwise specified. Normality will be assessed using Kolmogorov-Smirnov test. Differences of variables between baseline and over time within and between interventions will be tested by 2-way ANOVA with repeated measures on one factor in combination with Tukey post hoc tests.

For all statistical analyses, p values less than 0.05 will be considered to have statistical significance. Statistical analyses on metabolic (blood glucose, lipids, hormones and HOMA), inflammatory, dietary markers, and anthropometry data will be performed by Statistical Package for Social Sciences (version 16.0; SPSS, Inc.).

The effect of intervention and of time (inter-group and intra-group differences) as well all the possible associations between monitored parameters will be assessed in the whole population enrolled as well as in subgroups of subjects:

- defined on the basis of baseline (after run-in) consumption of fruit and vegetables (<3 servings/day)
- stratified for the response to one or more of metabolic parameters as well as for the compliance to the dietary intervention (assessed by the change of nutrients, specific metabolites or food categories over time).

Gut microbiome analysis will be carried out by high-throughput sequencing methods, based on both amplicon and shotgun approaches, in order to detect changes in the taxonomic and functional composition possibly driven by the intervention. Sequence data will be analyzed using the most up-to-date software available at the moment of the analysis. Inter-group and intra-group differences in microbial taxonomic composition, microbial pathways or specific genes, species or gene richness will be evaluated by pair-wise t-tests, Wilcoxon and/or Friedman tests, as appropriate.

Strain-level diversity analysis will be also explored on abundant microbial taxa by using the most appropriate tools for pangenome reconstruction from metagenomic data.

Correlation of variations in the gut microbiome with changes in the metabolome (urinary, blood and fecal), in metabolic, inflammatory and anthropometric parameters or dietary data will be defined using Spearman's, Pearson's and/or Kendall's correlations.

The same analyses will be carried out on the whole population, as well as in subgroups of subjects:

- defined on the basis of baseline (after run-in) consumption of fruit and vegetables (<3 servings/day)
- stratified for the response to one or more of metabolic parameters as well as for the compliance to the dietary intervention (assessed by the change of nutrients, specific metabolites or food categories over time).

Appropriate algorithms of clustering or statistical models will be used to define subgroups of MedD subjects who will respond better to the intervention (responders) based on the variation in specific metabolic, inflammatory or dietary markers.

All the analyses reported above will be repeated comparing "responders" subjects with either "non-responders" or ContD groups.

Statistical analyses and data visualization will be carried out in R environment (<http://www.r-project.org>).

6. References

1. Sofi, F., Abbate, R., Gensini, G. F., & Casini, A. (2010). Accruing evidence on benefits of adherence to the Mediterranean diet on health: an updated systematic review and meta-analysis. *The American journal of clinical nutrition*, 92(5), 1189-1196.
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3. Vitaglione, P., Mennella, I., Ferracane, R., Rivellese, A. A., Giacco, R., Ercolini, D., et al. (2014). Whole-grain wheat consumption reduces inflammation in a randomized controlled trial on overweight and obese subjects with unhealthy dietary and lifestyle behaviors: role of polyphenols bound to cereal dietary fiber-. *The American journal of clinical nutrition*, 101(2), 251-261.
4. De Filippis, F., Pellegrini, N., Vannini, L., Jeffery, I. B., La Storia, A., Laghi, L., et al. (2016). High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. *Gut*, 65(11), 1812-1821.